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Towards the detection of dietary cereal processing through absorbed lipid biomarkers in archaeological pottery

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ABSTRACT

The uptake of cereal agriculture in the Neolithic is one of the most important processes in later human prehistory. However, in many parts of Europe, early evidence from pollen or macrofossils is scarce or inconclusive, and there are considerable ambiguities about timing, intensity and the mode of transition to agriculture in these regions.

An alternative approach is organic residue analysis, a technique that targets lipids preserved in the walls of unglazed ceramic pots used for storage and processing of foodstuffs. By analysing the molecular and isotopic composition of absorbed lipid residues, many different food items and processing techniques can be detected and distinguished. However, this approach is by-and-large limited to animal-based food sources, and despite their importance, many plant-based food items including cereals are currently not accessible with this approach.

For a better understanding of the behaviour of cereal lipids, cooking experiments were conducted and the uptake of cereal-specific compounds such as alkylresorcinols and plant sterols into the ceramic matrix was investigated using a new sensitive method based on GC-Q-ToF-MS. Furthermore, changes in the lipid composition through post-burial degradation was assessed by incubation of potsherds dosed with cereal lipids at 35 °C in compost. The cooking experiments showed that only small quantities of cereal lipids are liberated, but additional lipid sources (meat) can increase the transfer of cereal biomarkers into the ceramic matrix. Anoxic degradation conditions allowed for twentyfold higher levels of alkylresorcinols and twofold higher levels of plant sterols after 20 weeks compared to oxic conditions. Therefore, samples from anoxic burial environments should be targeted and high sensitivity methods are a necessity to detect the trace amounts of cereal-specific biomarkers. To test the applicability of these biomarkers for archaeological pottery, organic residues from ten coarse ware vessels from an anoxic burial context at Vindolanda were analysed. Plant sterols and stanols were detected in three sherds, and two of the sherds also contained traces of alkylresorcinols.

1. Introduction

Cereal is a collective term for grasses with grains used for nutritional purposes. Today they are a staple food all over the world with annual production exceeding 2.5 billion tons (Food and Agriculture Organisation of the United Nations, 2017). The domestication of wild plants and the start of agriculture is regarded as one of the greatest and most important achievements in later human prehistory (Brown et al., 2009). The domestication of wild cereal ancestors was initiated in multiple parts of the world over 10 000 years ago, including the so-called “Fertile Crescent” of the Near East (Brown et al., 2009; Lougas et al., 2007). From there, agriculture spread westwards, reaching Europe about 6000 BCE, the British Isles and Northern Europe around 4000 and 3000 BCE, respectively (Lougas et al., 2007). However, there is still a significant debate about the timing and mode of uptake of cereal processing in certain regions (Rowley-Conwy, 2004; Lahtinen and Rowley-Conwy, 2013; Behre, 2006; Price, 2000).

The most common lines of evidence to follow and detect cereal usage and agricultural technology in prehistory are based on pollen analysis, macrofossils such as charred grains or agricultural artefacts such as sickles. All palaeodietary proxies, however, carry inherent biases which have created controversies regarding the role of cereal agriculture in certain regions (Behre, 2006; Brown,

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2007; Lahtinen and Rowley-Conwy, 2013; Stevens and Fuller, 2012). An alternative approach allowing the direct evidence of a commodity as an exploited resource is lipid residue analysis of archaeological potsherds (Evershed, 2008a, 2008b). This approach is based on the premise that storage and preparation of food items in unglazed ceramic pottery facilitates the transfer of constituents from the food into the ceramic matrix. Of these constituents, mainly DNA, proteins, carbohydrates and lipids, the latter have the highest chance of survival over archaeological timescales (Evershed, 2008b). However, usually not the original lipid compound is recovered but a characteristic degradation product (for example ω-(*o*-alkylphenyl)-alkanoic acids formed from heated polyunsaturated fatty acid in fish lipids), which can be used as a proxy for certain lipid sources (Evershed, 2008a, 2008b). By analysing the lipids, their characteristic degradation products and their isotopic signatures, information about the original composition and the lipid source can be gathered. This can then be used to reconstruct dietary and cultural habits as well as food processing technologies of past communities (Roffet-Salque et al., 2015; Salque et al., 2013; Cramp and Evershed, 2014; Cramp et al., 2015; Roffet-Salque et al., 2016).

To this day this approach is by-and-large limited to the analysis of animal-based food items. Despite their importance cereals and other plant-based food items are hard to detect in the archaeological record using organic residue analysis. The lipid content of plant-based food generally is at least tenfold lower, and the contribution will be likely masked by concomitant animal fat and therefore almost invisible (Colonese et al., 2017). Furthermore, only for very few plant-based food items (members of brassica family, maize kernels) have robust biomarkers been proposed and tested. Recently, Heron et al. showed the applicability of the triterpenoid miliacin as a biomarker for broomcorn millet processing in bronze age pottery (Heron et al., 2016). Furthermore, Colonese et al. identified wheat and rye kernels in an amorphous residue found in a well-preserved Bronze Age wooden container (Colonese et al., 2017). In addition to macrobotanical and proteomics data, they were able to detect alkylresorcinols and plant sterols, which are both well-known minor compounds of cereals (Ross et al., 2003; Piironen et al., 2002).

While this demonstrates that these compounds can be preserved under favourable conditions it raises the question why molecular evidence of cereals in archaeological pottery has been elusive so far. One explanation could be that the compounds are not liberated from the grains during cooking and thus are not absorbed into the ceramic matrix in appreciable quantities in the first place. Another explanation could lie in a high susceptibility to microbial degradation during millennia in the soil.

To shed more light on these questions, cereals were cooked multiple times in replica Neolithic pottery and absorption of cereal lipids into the ceramic matrix was quantified using a new and sensitive approach based on gas chromatography coupled to quadrupole-time-of-flight mass spectrometry (GC-Q-ToF-MS). Furthermore, potsherds containing cereal lipids were incubated at elevated temperatures in the laboratory to simulate accelerated microbial decay in the soil. With these experiments, we wished to evaluate how and under which conditions cereal lipids could be detected in archaeological pottery. The new method was then applied for the analysis of ten potsherds from a Roman cavalry barrack excavated at Vindolanda (105–120 CE).

2. Material and methods

2.1. Chemicals, standards and samples

Dichloromethane (DCM), *n*-hexane, methanol and chloroform (all HPLC grade) were from Rathburn Chemicals (Walkerburn, UK). Pyridine (>99%), the silylating agent consisting of N,O-Bis(trifluoroacetamide)/trimethylchlorosilane (BSTFA/TMCS) 99:1 (v/v), tetratriacontane (C34, >98%), and methyl heptadecanoate (17:0-ME, >99%) were from Sigma-Aldrich. An authentic standard of 5-*n*-docosoylresorcinol (AR-22, >99%) was from ReseaChem (Burgdorf, Switzerland).

Commercial compost, milk and pork was obtained in Bristol, UK and cereal samples (organically produced grains of spelt, rye and pot barley) were bought online. Cooking experiments were performed using modern replica pots made up from a clay/sand mixture (3:1) which were fired under 1000 °C (The Pot Shop, Lincoln/UK). After firing care was taken to avoid contamination from skin lipids. The pots (10 cm high, 11 cm diameter, 1 cm wall thickness) had a volume of about 500 mL. Ten coarse ware vessels (including one mortarium) from excavations at Vindolanda/UK were analysed. Sherds were selected from the rim part of the vessels to maximise lipid recovery. The vessels were from a drain between three rooms of a period IV cavalry barrack (ca. 105–120 CE). The conditions of this context were anaerobic, and as such, remarkable preservation of organic remains such as wooden writing tablets and textiles was observed.

2.2. Cooking experiments

About 80 g of cereals (equal parts of spelt, rye and barley) was placed together with 250 mL of (in-lab produced) ultrapure water into a replica pot and heated on a laboratory mantle heater. The pot was left to simmer for about 1 h until the kernels were soft. The mixture was stirred every 10–15 min and water lost from evaporation was replaced. Afterwards, the pot was emptied and cleaned with water and re-used for cooking until ten repeated cooking steps were performed. The same experiment was performed with two additional pots (Samples W1-3).

In two further experiments, 80 g of cereals, that had been ground in a coffee grinder (W4) and 80 g of cereals, which had been soaked in water for 12 h to soften them (W5), were simmered in a pot using 250 mL water for 1 h (five repeated steps).

Analogous to the first experiment 80 g of cereals were cooked in one pot in 250 mL of milk (3.5% fat) for ten repeated steps (Sample M).

Finally, one pot was used to cook cereals (80 g) together with pork shoulder (100 g, 10% fat) in 250 mL of water for ten repeated steps (Sample P).

Portions of the pots from the rim area (waterline) were removed using a hammer and a cleaned chisel, cleaned using a modelling drill and extracted as described below.

To assess the extractability of cereal lipids by water 20 g of cereals were refluxed in 200 mL of water for 22 h. After cooling, the lipids were extracted from the aqueous phase using 3 × 50 mL of DCM. The DCM phase was dried over sodium sulphate, the solvent was evaporated, and an aliquot of the re-dissolved sample was trimethylsilylated and analysed by GC/MS.

2.3. Dosing of potsherds with cereal lipids

A total of 12 g of the cereal mixture used for cooking experiments was extracted in 6 subsamples using 2 × 10 mL chloroform/methanol 2:1 (v/v) under sonication (20 min). After centrifugation the organic phases were combined and evaporated using a gentle stream of nitrogen, then re-diluted in 5 mL chloroform/methanol 2:1 (v/v). All total lipid extracts (TLEs) were combined and made up to 100 mL with chloroform/methanol 2:1 (v/v) and an aliquot of the solution was analysed by GC and GC-MS. The concentration of the TLE was about 1 mg/mL (GC-FID).

Sherds from an unused replica pot were dosed with the TLE by 6 × 10 min sonication. Every 10 min the sherds were moved in the TLE to ensure an equal distribution. The sherds were then left to dry overnight, and three sherds were cleaned and extracted as reference, while the remaining sherds were used for degradation experiments (see below).

2.4. Degradation experiments

Degradation experiments were performed in a laboratory incubator at 35 °C under oxic and anoxic conditions. For the oxic experiment a cleaned and furnaced 1 L glass bottle was filled about 1/4 with compost and cereal-lipid dosed potsherds were placed together with more compost into the bottle. About 20 mL of water was added to provide moisture and the bottle was closed with a bung of furnaced glass wool. To account for water loss through evaporation about 10 mL of water was added every week. Samples (three sherds) from the bottle were taken after 1, 2, 4, 10, and 20 weeks and analysed for their lipid composition.

For the anoxic experiment 100 mL glass bottles were half-filled with compost and purged with nitrogen. Under continued purging three dosed potsherds and more compost were added to the bottles, about 5 mL of water was added, and the bottles were tightly closed using rubber bungs with glass fermentation tubes extending through the bung.

One bottle containing three sherds was removed from the incubator after 1, 2, 4, 10, and 20 weeks and the sherds were analysed for their lipid composition.

Three blank sherds in compost and three dosed sherds without compost were incubated together with the samples as blank controls.

2.5. Lipid extraction from potsherd samples and cereals

Samples from cooking and degradation experiments were cleaned carefully using a modelling drill and crushed to a fine powder using a DCM washed mortar and pestle. After addition of 40 μg of internal standard tetratriacontane (C34) the total lipids were extracted from 2 g of powder using 2 × 10 mL of chloroform/methanol 2:1 (v/v) as described before (Cramp et al., 2014).

Cereals (5 g) were dried at 100 °C overnight and ground to a fine powder using a DCM cleaned mortar and pestle. Two samples (1 g) of the powder were spiked with 0.4 mg of the internal standard C34 and extracted under sonication (20 min) using 2 × 10 mL chloroform/methanol 2:1 (v/v) and 10 mL *n*-hexane. After centrifugation, the organic phases were combined, and the solvent was removed using a gentle stream of nitrogen. The residue was re-diluted in 5 mL chloroform/methanol 2:1 (v/v) and stored at −20 °C.

2.6. Sample clean up and derivatisation

Aliquots of the TLEs were applied to a small glass column (1 cm i.d.) filled with 0.5 g activated silica (conditioned with 5 mL chloroform/methanol 2:1 (v/v)) and eluted with 5 mL chloroform/methanol 2:1 (v/v). The solvent was transferred to a furnaced 1.75 mL vial and blown to dryness using a gentle stream of nitrogen.

The residue was re-dissolved in 25 μL dry pyridine and 50 μL of the silylating agent (BSTFA/TMCS 99:1, v/v), the vial was sealed and heated at 70 °C for 1 h. Afterwards, the silylating agent was removed using a gentle stream of nitrogen (to avoid contaminating the GC column), the residue was re-dissolved in 0.5 mL of *n*-hexane and after addition of the second internal standard methyl heptadecanoate (6 μg) the solution was used for GC-FID and GC-Q-ToF-MS analysis.

2.7. Analysis of archaeological samples and enrichment of alkylresorcinols from archaeological samples

Lipids from archaeological ceramic samples were extracted as described in section 2.5 for ceramics from cooking experiments, the final volume was 2 mL. An aliquot of 0.5 mL was cleaned using a silica micro column (section 2.6) and the residue was re-dissolved in 0.5 mL *n*-hexane. An aliquot (100 μL) was derivatised as described before, re-dissolved in 100 μL *n*-hexane and screened by GC and GC-MS.

The remaining solution (400 μL) was spiked with 5 ng AR-22 and given on a Isolute NH2 SPE column (Biotage, 500 mg, 6 mL) that had been pre-conditioned with 6 mL *n*-hexane. Interfering lipids were eluted with 6 mL *n*-hexane and 6 mL DCM before ARs were eluted with 6 mL methanol. The solvent was removed using a gentle stream of nitrogen and the residue was trimethylsilylated as described before. After removal of the silylation agent the residue was re-dissolved in 50 μL *n*-hexane and analysed using GC-Q-ToF-MS.

2.8. GC-FID analysis of lipid extracts

TLEs after trimethylsilylation were analysed using a 7890 GC-FID (Agilent, Santa Clara, CA/USA) equipped with a 7683B autosampler. A 15 m, 0.32 mm i.d., 0.1 μm film thickness DB-1HT column (100% dimethylpolysiloxane, Agilent, Santa Clara, CA/USA) was installed in the oven. Injections (1 μL) were performed using the cool-on-column inlet and helium was used as carrier gas at a constant flow of 4.0 mL/min. The oven was programmed as follows: After 2 min at 50 °C, the temperature was increased at a rate of 10 °C/min to 350 °C, which was held for 10 min. The temperature of the inlet was set to follow the oven temperature and the FID temperature was set to 350 °C while maintaining flow rates of hydrogen, air and nitrogen at 30, 400 and 27 mL/min, respectively.

2.9. GC-Q-ToF-MS analysis of lipid extracts and alkylresorcinols after NH2 SPE enrichment

TLEs from cereals and reference experiments were analysed using a 7890/7200B GC-Q-ToF-MS (Agilent, Santa Clara, CA/USA). A 15 m, 0.25 mm i.d., 0.1 mm film thickness ZB-5HT Inferno column (Phenomenex, Torrance, CA/USA) was used for the separation. Samples (1 μL) were injected using a 7693 autosampler and a cool-on-column inlet (set to follow the oven temperature). Helium was used as carrier gas with a flow rate of 1.5 mL/min. The oven was programmed as follows: After 2 min at 55 °C the temperature was raised to 220 °C at a rate of 10 °C/min and immediately afterwards to 350 °C at 20 °C/min. This final temperature was held for 13 min. The temperature of the transfer line, ion source and quadrupole were set to 350, 300 and 180 °C, respectively. Data were recorded from *m/z* 50–1050 with a rate of 5 scans/s in the *Extended Dynamic Range* mode.

For analysis of alkylresorcinols after SPE enrichment a 50 m, 0.32 mm i.d., 0.17 μm film thickness HP-1 column (Agilent, Santa Clara, CA/USA) was installed and samples (1 μL) were injected using the Multimode Inlet in splitless mode. Helium was used as carrier gas with a flow rate of 1.5 mL/min. The oven was programmed as follows: After 2 min at 55 °C the temperature was raised to 320 °C at a rate of 20 °C/min. This final temperature was held for 10 min. The temperature of the transfer line, ion source and quadrupole were set to 320, 230 and 150 °C, respectively. Data were recorded from *m/z* 50–1050 with a rate of 5 scans/s in the *Extended Dynamic Range* mode.

3. Results and discussion

3.1. Composition of lipids in the cereal sample

Extraction of the lipids from the rye, spelt, and barley mixture and GC-FID analysis enabled the detection of multiple compounds in varying contributions at a total concentration of 9.4 mg/g d.m (GC-FID). The major peaks were triacylglycerols (TAGs), with C54 and C52 TAGs dominating and minor contributions of C50, C56 and C48 TAGs (Fig. 1). The TAGs accounted for about 70% of compounds (calculated from total peak areas) present in the sample. Free fatty acids (C16:0, C18:2, 18:1 and 18:0) contributed about 5% (calculated from peak areas) to the total lipids. Noteworthy, there were also non-lipid compounds in the extract, which were identified as sugars (mono-, di-, and trisaccharides) by GC-MS. A reference analysis of the sample before silica column confirmed high amounts of these compounds in the extracts, and a fraction was eluted with the lipids from the silica column.

In addition, GC-MS allowed the identification of several alkylresorcinol isomers in the samples (total content of ~400 μg/g d.m.), which are known marker compounds for cereals and could even be used to discriminate different cereals (Ross et al., 2003; Ziegler et al., 2015). The main isomers were 5-*n*-heneicosylresorcinol (AR-21, relative intensity 100) and AR-19 (86), with minor contributions of AR-17 (33), AR-23 (26), AR-25 (18), AR-15 (2), and AR-27 (1) (Fig. 2a,c). The distribution of isomers was corresponding with a mixture of rye and spelt (Ross et al., 2003). The contribution of ARs from barley was negligible due to the almost twentyfold lower concentrations of ARs in barley (Ross et al., 2003). The saturated AR isomers were accompanied by unsaturated isomers, which eluted slightly earlier than the saturated homologues and could be identified based on their molecular ion (Ross et al., 2004). The presence of these unsaturated compounds particularly in rye had been described before (Ross et al., 2001). Further minor lipids were identified in the form of tocochromanols as well as sterols and stanols, with the sterols being present as free sterols, glycosides as well as esterified to fatty acids. In particular the plant sterol compounds and alkylresorcinols were of interest, since they are not occurring in animals in significant concentrations and would be strong candidates as biomarkers.

3.2. Uptake of cereal lipids into ceramic matrix

3.2.1. Cooking of cereals in water

Extraction and GC-FID analysis of each two sherds from samples

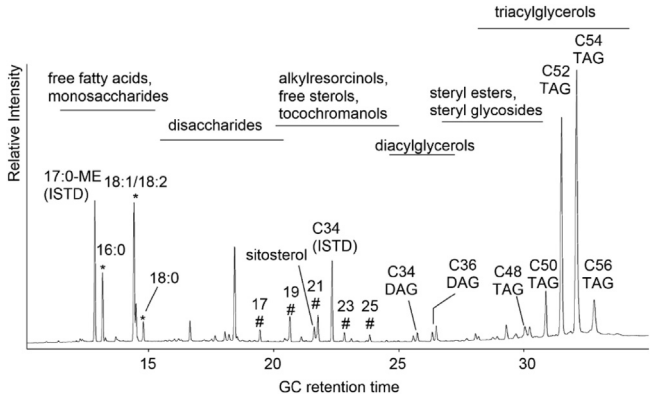

Fig. 1. GC-FID chromatogram of the lipid extract from a mixture of spelt, rye and barley after trimethylsilylation showing the elution of fatty acids (*), alkylresorcinols (#), diacylglycerols (DAG) and triacylglycerols (TAG).

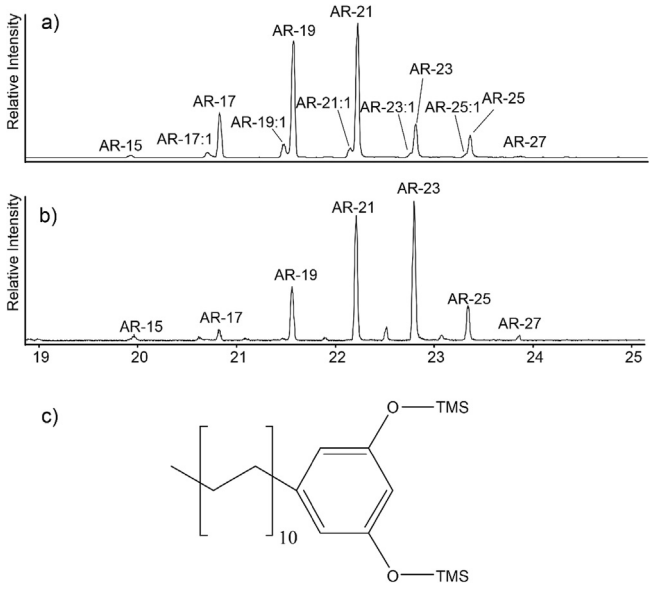

Fig. 2. GC-Q-ToF-MS Extracted Ion Chromatograms (*m/z* 268.1315) showing a) the alkylresorcinol (AR) isomer distribution in the cereals used for cooking experiments, b) in the extract from the pot after 10 cooking steps (sample W1), and c) the structure of AR-21 as di-TMS ether.

W1-3 taken from the rim region of the pots showed that only very little lipids had been incorporated into the pots. The total lipid concentrations in these samples was <10 μg/g and therefore close to the usually applied threshold for reliably interpretable lipid concentrations of 5 μg/g (Evershed, 2008a). In this context, it has to be considered that we could only detect GC-amenable lipids, and abundant polar lipids in cereals (such as phospholipids and glycolipids (Morrison, 1978)) potentially present in the TLEs were not detected with this method. In contrast, abundant non-lipid peaks were present in the GC chromatograms, which could be identified by GC-MS as monosaccharides.

Samples had been taken from the rim region of the pots because previous experiments revealed highest lipid concentrations in that region for water-cooked foodstuffs (Charters et al., 1997; Charters, 1996). Resampling of two further sherds from the middle and base of a pot did not yield higher lipid concentrations. Noteworthy, analysis of the TLEs by GC-Q-ToF-MS enabled the detection of alkylresorcinols as well as traces of free and esterified sterols. However, concentrations of ARs were very low (~0.2 μg/g). Furthermore, the isomer pattern of the ARs was notably different from the cereal sample used for cooking. In the sherds from the cooking experiment, AR-23 (relative intensity 100) was the main isomer followed by AR-21 (90), AR-19 (40), AR-25 (25), AR-17 (8), AR-15 (5), and AR-27 (3) (Fig. 2b) and the unsaturated isomers were virtually absent. In contrast to this, extracts from potsherds dosed with the cereal TLE (see section 2.3) showed the original isomer distribution, which means that this pattern change was most likely linked to different extraction and absorption during the cooking process. This has important implications, as it drastically limits the possibilities to use the AR distribution from cooking pots to identify specific cereals.

Two possibilities for the low lipid uptake into the pot sherd samples were considered. Either water was not able to extract the lipids from the kernels or they were not incorporated into the ceramic fabric. Further experiments tried to improve the extractability of the lipids. By grinding the cereals (sample W4) the surface should be increased and a better access to the lipids should be achieved. Similarly, softening of the kernels prior to cooking

(sample W5) was expected to offer better extraction of the lipids. Yet, analysis of samples from these pots showed that these measures did not have any notable effects and lipid concentrations were still <10 μg/g. Even refluxing cereals for 20 h in water released only little lipids to the cooking water. The amount of lipids which was extracted with DCM from the water amounted to about 10 μg/g cereal, which was about 0.1% of the amount (9.4 mg/g d.m.) which could be extracted using $CHCl_3$/MeOH 2:1 (v/v). This showed that the primary problem was the low extraction efficiency of water for cereal lipids. In previous cooking experiments with lamb meat and brassica leaves considerably higher levels of absorbed lipids (21.8 mg/g and 0.26 mg/g, respectively) were found (Evershed, 2008a). In contrast to these commodities, where the lipids are on the surface and were washed off by the hot water, cereal lipids are mostly inside the grain, often associated with starches or proteins (Morrison, 1978, 1988; Ruibal-Mendieta et al., 2002). This likely trapped the lipids and hampered their extraction from the cereals during the cooking process.

3.2.2. Cooking of cereals together with animal-derived lipids

In light of the very limited extraction of cereal lipids by water, we tested if lipids present in the water could enhance the extraction by acting as a carrier and extraction medium for the cereal lipids. Cooking in milk (3.5% fat) instead of water (sample M) led to a significant uptake of lipids (~0.6 mg/g) into the ceramic matrix. The major peaks were C26-C54 TAGs with a distribution typical for milk fat (Dudd and Evershed, 1998; Jensen, 2002) accompanied by monosaccharides and minor contributions of free fatty acids, and cholesterol. While this showed that the ceramic could absorb considerable amounts of lipids (including sterols in the form of cholesterol) we could not find plant sterols or other clearly cereal-derived compounds in the extracts by GC-FID analysis. GC-Q-ToF-MS analysis allowed the detection of alkylresorcinol isomers at a total level of 0.2 μg/g, which is similar to the level observed in the W samples. Similarly, plant sterols could only be detected in minute traces.

During the cooking experiments using pork shoulder (10% fat) liberated lipids were clearly visible as a layer floating on top of the water. Accordingly, lipid concentrations found in samples from the rim of the pot (~4 mg/g) were tenfold higher than from the body and the base (0.4–0.6 mg/g). This vertical gradient of lipid concentrations after boiling of fat-rich food in water had been observed before (Charters et al., 1993). Similar to the M sample, plant sterol compounds could only be detected in traces. In contrast to this ARs were present at a level of slightly over 1 μg/g, which is fivefold higher than in the W samples.

This finding supports the hypothesis that non-cereal lipids could work as a carrier and enhance the extraction of ARs from the cereal grains and their uptake into the ceramic matrix of the pots. For archaeological samples, this scenario is quite realistic as it is plausible that several food commodities would have been cooked simultaneously in the same cooking pot. Pottage or stews containing cereal grains and meat as well as other vegetables are well-known from historical European recipe books (e.g. The English Huswife (Markham 1615)), and as traditional dishes around the world.

Although in these experiments only low transfer of cereal biomarkers into the pottery matrix was observed, this does not exclude their potential presence in higher abundance in archaeological pottery. In the laboratory, only a limited number of cooking steps could be realised, while archaeological pots could have been used daily for many years, in that way accumulating several thousand cooking steps and leading to significantly higher lipid concentrations. To account for these potentially higher lipid yields and to be able to investigate their degradation behaviour, potsherds dosed with TLE solutions were used for the microbial decay experiments.

3.3. Degradation of cereal lipids under laboratory incubation conditions

3.3.1. General trends of oxic and anoxic degradation

Dosing of blank sherds in the TLE (see section 2.3) facilitated an uptake of ca. 190 μg/g lipids into the ceramic fabric. The composition of the lipids in the sherds corresponded well with the composition of the TLE used for dosing.

Under oxic conditions, degradation occurred rapidly and after one week less than half of the initial amount could be found in the sherds (ca. 80 μg/g, Fig. 3). Responsible for this was a strong decrease of TAGs. Although there was a slight increase of C34 and C36 diacylglycerols (particularly 1,2 isomers), lipids were mineralised rapidly and there was no significant accumulation of hydrolysis products. Lipid degradation continued rapidly and after 20 weeks under oxic conditions, triacylglycerols had been almost completely degraded and only very few peaks were detected using GC-FID (total lipids ca. 6 μg/g) (Fig. 4a).

In contrast to the oxic degradation the anoxic degradation occurred slower and after one week the lipid content was significantly higher at about 130 μg/g (Fig. 3). While the decrease in total lipids could be attributed to a decrease of TAGs, a higher proportion of free fatty acids as well as mono- and diacylglycerols was observed. This hinted at more hydrolytic activity compared to the oxic degradation pathway. Accordingly, even after twenty weeks the total lipid concentration was still 35 μg/g with clearly identifiable TAGs (Fig. 4b). These general results were in accordance to observations from previous degradation experiments (Evershed, 2008a; Dudd et al., 1998; Charters, 1996).

3.3.2. Stability of cereal biomarkers

GC-Q-ToF-MS analysis was used to investigate the fate of individual lipid compounds during the degradation process, with a special focus laid on alkylresorcinols and plant sterols.

Plant sterols proved to be quite resilient to microbial degradation in our experiments. Starting at levels of 4.5 μg/g in the dosed sherds (sum of free sterols, steryl glycosides and fatty acid esters), levels in the sherds were still at 1.6 μg/g after 20 weeks subjected to oxic degradation. Accordingly, about 35% of the plant sterol

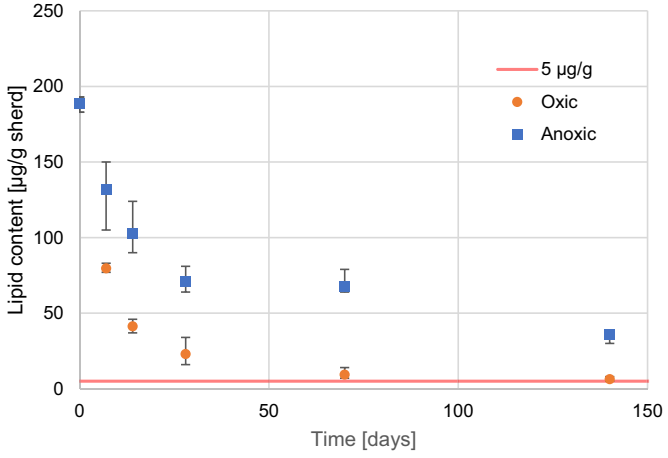

Fig. 3. Total lipid contents (determined by GC-FID) in potsherds dosed with cereal lipids and incubated at 35 °C under oxic (circles) and anoxic (squares) conditions in compost. Samples were analysed after 1, 2, 4, 10, and 20 weeks. Markers are averages of triplicate samples and error bars denote the range.

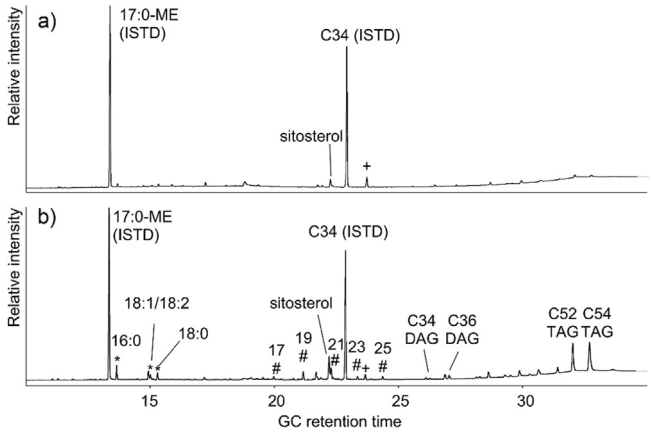

Fig. 4. GC-FID chromatograms (after trimethylsilylation) of the lipid extracts from potsherds after 20 weeks of incubation under a) oxic and b) anoxic conditions showing the elution of fatty acids (*), alkylresorcinols (#), diacylglycerols (DAG) and triacylglycerols (TAG). The peak marked with "+" is a plasticizer.

compounds were still present, while the total lipids dropped to 3% of the initial levels and at this point sterols were with the most prominent peaks in the chromatograms (Fig. 4a). Noteworthy, in the samples subjected to anoxic conditions plant sterols were still present at almost the initial levels (4 μg/g) after 20 weeks. Both sample groups showed traces of ergosterol which is a common sterol in several fungi (Weete et al., 2010) and was most likely taken up from the compost into the potsherd. The low degradation of plant sterols was rather surprising, since sterols are virtually absent in archaeological samples. In light of these results it appears more likely that sterols are degraded during the use of the pots instead of during post-use burial.

Compared with sterols, alkylresorcinols proved to be more susceptible to microbial degradation. While the dosed sherds initially contained AR levels of 24 μg/g, the levels dropped to 9 μg/g after one week of oxic incubation and after 20 weeks the levels had dropped to 0.2 μg/g. Higher levels of ARs were found in the samples incubated under anoxic conditions and about 20% of the initial amount (4.5 μg/g) were still present after 20 weeks of incubation. Interestingly, degradation under anoxic conditions did not alter the AR isomer pattern.

Both for sterols and ARs a strong influence of the degradation conditions (and with that of potential burial conditions) was observed with anoxic conditions leading to clearly higher levels of cereal biomarkers.

3.4. Analysis of potsherds from Vindolanda for cereal biomarkers

Ten sherds from the Roman fort of Vindolanda on Hadrian's Wall were selected to screen for the new biomarkers, because there is strong palaeobotanical and written evidence for cereal processing and consumption at this settlement (Pearce, 2002; Birley, 2002; Manning et al., 1997). Furthermore, the anoxic burial conditions should allow for good preservation of lipids in general and cereal biomarkers in particular. In fact, nine of the ten sherds yielded lipid contents over 5 μg/g (37–1240 μg/g, average 339 μg/g) (Table 1). In all nine sherds free C16 and C18 fatty acids could be detected and 6 samples also featured branched and odd-chain fatty acids. In all but one samples, intact triacylglycerols (C46-C56) could be detected, again proving the good lipid preservation conditions. Further lipid classes identified were monoacylglycerols (MAGs, C16 and C18) and DAGs (C32-C36) as well as wax esters (C40-C48) (Table 1).

Surprisingly, fully saturated stanols and (in lower quantities) respective sterols were detected in 4 samples (Nos 1,4, 9, 10), and three of these samples also featured steroidal ketones. While cholesterol and cholestanol could likely originate from modern post-burial contamination the plant sterols and stanols found in samples 4, 9 and 10 are very unlikely to be contaminants. Further evidence that these compounds are genuine sample components is found from the respective steroidal ketones, which have been observed as microbiological post-burial degradation products of sterols and intermediates in the formation of stanols (Evershed and Connolly, 1994; Mackenzie et al., 1982). Although sterols are very rarely encountered in archaeological samples, the anoxic burial conditions encountered in this site very likely enabled their preservation (see 3.3.2).

Noteworthy, in two of the samples (Nos 4 and 10) we were

Table 1
Lipid content [μg/g] and lipid composition in the samples from Vindolanda. Abbreviations: FFA, free fatty acid; MAG, monoacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol; AR, alkylresorcinol.

Sample No	Sample Name	Lipid content [μg/g]	Lipid composition[a]					
			FFA	MAG	DAG	TAG	AR	Miscellaneous other compounds
1	V17-94A-1	160	12:0, 14:0, br15:0, 15:0, 16:1, 16:0, br17:0, 17:0, 18:1, 18:0	C16, C18	C32, C34, C36	C48-C56	–	alcohol: C26-C32, wax ester: C40-C48, cholesterol, cholestanol
2	V17-94A-2	707	14:0, br15:0, 15:0, 16:1, 16:0, br17:0, 17:0, 18:1, 18:0	C16, C18	C32, C34, C36	C48-C54	–	
3	V17-94A-3	230	16:0, 18:0	C16, C18	C32, C34, C36	C48-C56	–	Wax ester: C42-C48
4	V17-94A-4	1240	14:0, br15:0, 15:0, 16:1, 16:0, br17:0, 17:0, 18:1, 18:0, 19:0, 20:0	C16, C18	C32, C34, C36	C46-C56	19, 21, 23, 25	cholestanone, cholesterol, cholestanol, campestanol, sitosterol, sitostanol, sulfur (S_8)
5	V17-94A-5	83	14:0, br15:0, 15:0, 16:0, br17:0, 17:0, 18:1, 18:0	–	–	C48-C54	–	wax ester: C40-C48, sulfur (S_8)
6	V17-94A-6	37	16:0, 18:0	–	–	–	–	–
7	V17-94A-8	<5	–	–	–	–	–	–
8	V17-94A-9	245	14:0, 15:0, 16:0, br17:0, 17:0, 18:1, 18:0	C16, C18	–	C48-C54	–	–
9	V17-94A-10	290	14:0, br15:0, 15:0, 16:1, 16:0, br17:0, 17:0, 18:1, 18:0	C16, C18	C34, C36	C48-C56	–	cholestanone, cholestanol, campestanol, sitostanol
10	V17-94A-11	60	16:0, 18:0	–	–	C48-C56	19, 21, 23, 25	alcohol: C18, C24-C32, wax ester: C42-C48, cholestanone, cholestanol, sitostanone, sitostanol, sulfur (S_8)

[a] TLEs were screened using the GC-MS set up described in Roffet-Salque et al. (2015).

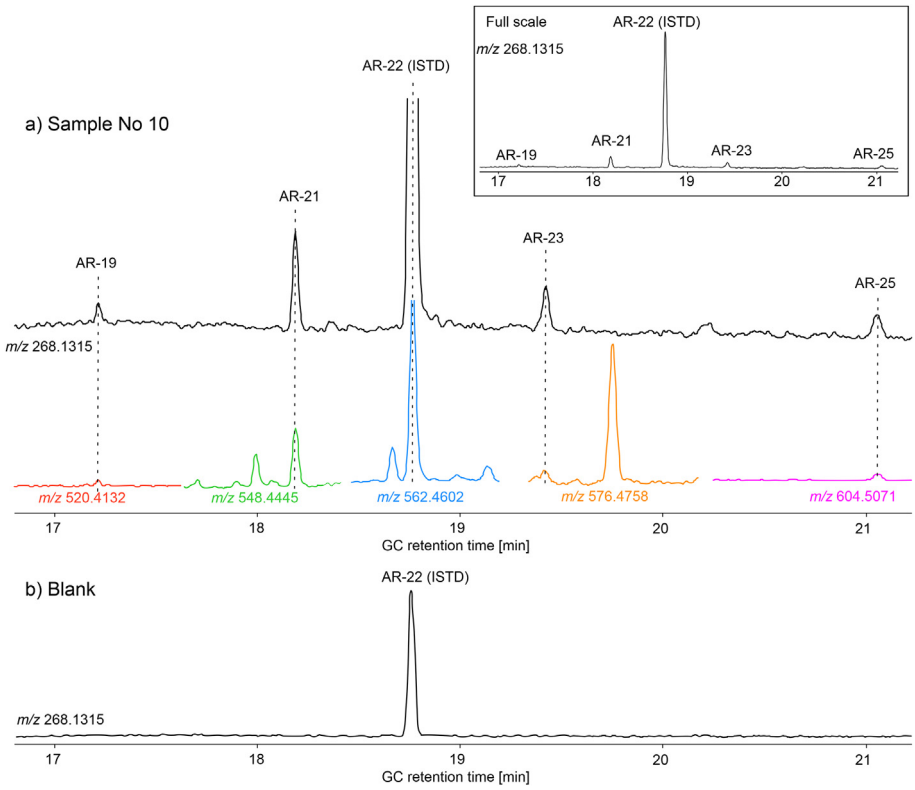

Fig. 5. GC-Q-ToF-MS Extracted Ion Chromatograms (smoothed) of *m/z* 268.1315 (all ARs) for a) sample No 10 (zoomed, full view in insertion top right) and b) a blank sample after NH2 SPE enrichment and trimethylsilylation. Detailed view in a) also displays excerpts of the Extraction Ion Chromatograms for molecular ions of AR-19 (*m/z* 520.4132, red), AR-21 (*m/z* 548.4445, green), the internal standard AR-22 (*m/z* 562.4602, blue), AR-23 (*m/z* 576.4758, orange), and AR-25 (*m/z* 604.5071, pink). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

able to detect very small concentrations of ARs (<1 ng/g) after SPE enrichment, with AR-21 being the major isomer alongside smaller contributions of AR-19, AR-23, and AR-25 (Fig. 5a). Identification of ARs was achieved through GC elution relative to the internal standard AR-22 and the extracted ion chromatograms for the AR base peak (*m/z* 268.1315) and the molecular ions for AR-19 (*m/z* 520.4132), AR-21 (*m/z* 548.4445), AR-23 (*m/z* 576.4758) and AR-25 (*m/z* 604.5071) (Fig. 5a). Noteworthy, analytical blanks for the extraction and the SPE enrichment did not show any relevant peaks, thus providing evidence that these compounds were not from carry-over or minor contaminants in the AR-22 standard (Fig. 5b).

Two of the samples (4 and 10) contained both plant sterol compounds as well as alkylresorcinols, thus showing that these compounds indeed can survive in archaeological pottery under favourable burial conditions. The presence of both these compound classes strongly hints that these containers have in fact been used for processing of cereals in some way.

4. Conclusions

Our findings indicate that the chances of detecting preserved cereal biomarkers depends both on the food preparation techniques used and the burial conditions. It is unlikely that high levels of cereal biomarkers would be found in pots that had been solely used to water-cook cereals, since the uptake of cereal biomarkers in the ceramic matrix is very limited. On the other hand, additional fat could facilitate significantly higher levels in the pots, but even then, levels will be in most cases <<1 μg/g. Therefore, these minor contributions will most likely only be detected by targeted approaches, e.g. by using Selected Ion/Reaction Monitoring (SIM/SRM) methods or by using extracted ion traces in high resolution mass spectrometers (this study). More research is needed to optimize extraction and enrichment steps for both plant sterols and alkylresorcinols to enable their reliable identification in samples. Furthermore, sampling strategies should take the burial conditions into account and favour assemblages from anoxic burial conditions, as seen in the samples from Vindolanda analysed in this study. Thus, the combination of targeted sampling with adequate extraction and sample preparation as well as high sensitivity analysis provides optimal chances for the detection of the ever-elusive cereal lipid biomarkers in archaeological pottery.

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